

Filed: January 14, 2000

Title: COMPOSITIONS AND METHODS FOR NONENZYMATIC LIGATION OF OLIGONUCLEOTIDES AND
DETECTION OF GENETIC POLYMORPHISMS

Please replace the paragraph beginning at page 26, lines 1-12, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B2 The stability of the 5'-iodothymidine in comparison to the 5'-tosylthymidine was analyzed by thin layer chromatography under varied conditions. Results showed that the tosynucleoside in concentrated ammonia (55°C) has a half-life of less than 1 hour, whereas the iodonucleoside has a half-life of about 7 hours. When treated at room temperature for 24 hours (conc. NH₃) the tosynucleoside is >90% degraded, while the iodonucleoside is <2% degraded. The stability of the iodide in oligonucleotides was also analyzed by reverse-phase HPLC. Chromatograms revealed that the iodide (in the sequence 5'-I-TTCACGAGCCTG)(SEQ ID NO:3) has a half-life of >4 days in conc. NH₃ at 23°C, similar to that of the nucleoside alone. Based on the HPLC analysis we chose the following conditions for deprotection: concentrated ammonia, 55°C for 1 hour, followed by incubation at room temperature for 23 hours, or treatment at room temperature alone for 24 hours. It is anticipated that the iodide would also be stable to rapid deprotection conditions, although this was not explicitly tested.

Please replace the paragraph beginning at page 30, lines 4-9, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

B3 Oligonucleotides containing 5'-iodo- and 3'-phosphorothioate groups (20 mM) were incubated with 22 mM complementary splint oligomer in a pH 7.0 buffer (50 mM Tris•borate) containing 10 mM MgCl₂ at room temperature for 24 hours as described in Example I. The splint sequence used for the 20mer and 45mer DNAs was 5'-d(CTA GTC CAA AGT GCT CGG)(SEQ ID NO:4); for the hairpin sequence no splint was needed. Ligation products were isolated by preparative denaturing polyacrylamide gels.

Please replace the paragraph beginning at page 33, lines 2-13, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

BF We then investigated the susceptibility of this linkage ([5'-³²P]dGATCAGGT_pS TTCACGAGCCTG (SEQ ID NO:5), where "s" denotes position of sulfur in phosphorothioate linkage) to different exonuclease enzymes, specifically, the 3'-exonuclease activity of T4 DNA polymerase, snake venom phosphodiesterase (SVPDE) (a different 3' exonuclease), and calf spleen phosphodiesterase (CSPDE), which is a 5'-exonuclease. We found that sulfur causes a significant inhibition of T4 exonuclease activity. The pauses occur at sites one and two nucleotides 3' (prior) to the thioester rather than during the removal of the sulfur-containing nucleotide itself. We estimate the cleavage of the most resistant linkage to be inhibited by a factor of five to tenfold. Since the S-P bond is not expected to be cleaved by this enzyme (products with this enzyme are normally 5'-monophosphates), we surmise that this pause is due to unfavorable interaction of the enzyme with this sulfur, possibly because of the increased bond lengths or relatively poor hydrogen bond accepting ability of the sulfur relative to oxygen.

Please replace the paragraph beginning at page 35, lines 5-17, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

BS Finally, we examined the effects of one or two 5' bridging sulfur linkages on the thermal stability of DNA-DNA duplexes. This was tested first in the context of a 20mer duplex carrying one sulfur linkage in one strand (d(GAT CAG GT_pS TCA CGA GCC TG))(SEQ ID NO:6) and its unmodified 20mer complement), and a completely unmodified duplex of the same sequence was examined for comparison. Thermal denaturation studies (100 mM Na⁺, 10 mM Mg²⁺) showed that both cases were well-behaved, showing sharp transitions. T_m values were 68.8 °C for the sulfur-containing duplex and 71.5 °C for the unmodified duplex. A second case was then examined with 5'-S linkages in both strands of a duplex, this time using the dumbbell sequences

shown in Figure 7). Because of their high stability the denaturation studies were performed under low salt conditions (10 mM Na•PIPES, 1 mM EDTA). The results showed that the thermal stabilities of the modified duplex ($T_m = 82.8$ °C) and the unmodified one ($T_m = 83.3$ °C) are essentially the same. Thus, the 5'-bridging sulfur linkage causes very little destabilization of duplexes, at least for the cases studied here.

Please replace the paragraph beginning at page 45, lines 22-27, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Plasmid pT24 – C3 containing the c-Ha-*ras* 1 activated oncogene mutation at codon 12 (GGC-GTC) and pbc-N1, containing wild type c-Ha-*ras* were obtained from American Type Culture Collection. 300 bp regions including nucleotides – 53 (relative to the transcription initiation site) and + 244, of normal and activated Ha-*ras* genomic clones were PCR amplified using the primers 5'- GTG-GGG-CAG-GAG-ACC-CTG-TA(SEQ ID NO:7) (sense) and 5'- CCC-TCC-TCT-AGA-GGA-AGC-AG(SEQ ID NO:8) (antisense).

Please replace the paragraph beginning at page 56, lines 14-19, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Ligated oligonucleotides were characterized by their gel mobility and by electrospray mass spectrometry:

5'dGTG GGC GCC G-pO-TC GGT GT(SEQ ID NO:9)	calculated mass 5274.6; found, 5274
5'dGTG GGC GCC G-pS-TC GGT GT(SEQ ID NO:10)	calculated mass 5290.6; found, 5290
5'dGTG GGC GCC G-pSe-TC GGT GT(SEQ ID NO:11)	calculated mass 5337.6; found, 5337

Please enter the written Sequence Listing (17 pages) submitted herewith.